Cloning, sequence analysis and expression profiling of a heat shock protein 70 gene in *Tenebrio molitor* (Coleoptera: Tenebrionidae)

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Abstract: In order to study the mechanism of stress-resistance, a heat shock protein gene *hsp*70 (named as *Tmhsp*70), was cloned from the larvae of *Tenebrio molitor* by PCR and RACE method, and the mRNA levels in developmental stages were detected by using semi-quantitative RT-PCR as well. The results showed the full sequence of *Tmhsp*70 cloned is 2 282 bp in length containing a 115 bp 5′ untranslated region (5′ UTR) rich in adenine, a 1 935 bp open reading frame and a 232 bp 3′ untranslated region (3′ UTR) rich in adenine and thymine. It also has seven repeats of the heat shock element nGAAn in its 5′ UTR and a 22 bp Poly (A) tail in the 3′ UTR. The deduced heat shock protein named as TmHSP70 contains three signature motifs of HSP70, *i. e.*, IDLGTTYS, IFDLGGGTFDVSIL and IVLVGGSTRIPKIQQ as well as the terminal EEVD motif which is characteristic to cytoplasmic HSP70s. TmHSP70 has neither a signal peptide nor a transmembrane domain. It contains two main functional domains: a 42 kDa highly conserved N-terminal ATPase domain and a 18 kDa conserved C-terminal peptide-binding domain. The tertiary structure of ATPase domain is composed of two large globular subdomains and contains a nucleotide-binding core. Tertiary structure of the peptide-binding domain forms a sandwich of 2 four-stranded β-sheets and two α-helices, and includes a peptide-binding cavity. Furthermore, the expression of *Tmhsp*70 mRNA in *T. molitor* was characterized by heat-inducible and developmental-regulation feature. The overall increase in the levels of *Tmhsp*70 mRNA in different life stages when the larvae were exposed to 42℃ for 1 h, ranged from 1.4- to 26.9-fold on the basis of semi-quantitative RT-PCR analysis. At 25℃ *Tmhsp*70 mRNA expressed in 1-day old pupae was higher than that accumulated in other developmental stages, and after exposure to 42℃ for 1 h, *Tmhsp*70 mRNA expressed in 90-day old larvae became the most abundant, and was not only higher than that accumulated in 30- and 60-day old larvae but also higher than that accumulated in 15- and 30-day old adults. The results form a basis for further research on structure, function and expression regulation of HSPs from *T. molitor* as well as the relationship between HSPs and stress-resistance in the beetle.

Key words: *Tenebrio molitor*; heat shock protein (HSP); gene cloning; bioinformatics; mRNA expression; heat shock

1 INTRODUCTION

Heat shock proteins (HSPs) are important stress proteins that are closely associated with the adaptation of an organism to environmental changes and mainly divided into HSP100, HSP90, HSP70, HSP60, HSP40 and small molecule HSPs based on their molecular size (Schlesinger, 1990). HSPs have highly conserved structure and prominent biological functions (Borchiellini *et al.*, 1998; Chen *et al.*, 2005; Huang *et al.*, 2008). They primarily function as molecular chaperones to regulate biological functions of many kinds of proteins and a variety of vital processes. In particular, they are involved in the folding and transporting of nascent peptides and assembly, aggregation and degradation of certain proteins thereby playing a critical role in the maintenance of normal cell signaling and regulation of cell life (Sorger, 1991). Secondly, as important cell damage repair factors, HSPs repair denatured and inactivated proteins, protect the

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cytoskeleton (Brown *et al.*, 1996), and improve protective functions and antioxidant capacity of protective enzymes. They also avoid cell impairments by inhibiting denaturation and aggregation of intracellular proteins (Mestril and Dillmann, 1995). In addition, HSPs are involved in a variety of physiological and pathological processes such as apoptosis, heat tolerance, immunoregulation, oncogenesis and viral infection (Schlesinger, 1990). Since their discovery in the salivary glands of *Drosophila melanogaster* in 1962, the structure, function, expression and application of various kinds of HSPs have become research hotspots in life sciences. HSP genes have been cloned and sequenced from several insect species representing different insect orders (Konstantopoulou and Scouras, 1998; Landais *et al.*, 2001; Karouna-Renier *et al.*, 2003; Sonoda *et al.*, 2006; Jiang *et al.*, 2012). Traditionally, HSP genes are divided into inducible and constitutive groups (Lang *et al.*, 2000; Qin *et al.*, 2003). Genes in the inducible group are induced under stressful conditions, but return to a normal expression level when stress is removed (Lang *et al.*, 2000). Genes in the constitutive group are not stress-inducible, but are expressed at all times and are generally referred to as heat shock cognates (HSC) (Qin *et al.*, 2003; Karouna-Renier *et al.*, 2003). A variety of environmental stresses, including cold (Goto and Kimura, 1998), heat (Schlesinger, 1990), electric radiation (Ennamany *et al.*, 2008), ultraviolet irradiation (Trautinger, 2001), starvation (Yengkokpam *et al.*, 2008), anoxia (Prentice *et al.*, 2004), ischemia, organic pollutants, trace-metal exposure (Aït-Aïssa *et al.*, 2000), antibiotics, tissue wound, microbial infection (Jindal and Young, 1992) and tumor (Wei *et al.*, 1995) have been reported to induce HSPs in various organisms.

Insects are the most widely distributed biological groups on the earth and show very strong adaptability to various environmental conditions. Research on HSPs from insects has great value in understanding insects' stress-resistance. Different HSPs can be induced by different environmental factors, and the rapidly synthesized HSPs elevate insects' tolerance to unfavorable environments (Jong *et al.*, 2006; Rinehart *et al.*, 2006). The *hsp*70 and *hsp*90 genes have been reported to be up-regulated in response to cold and heat shock in several insects (Schlesinger, 1990; Goto and Kimura, 1998; Yocum, 2001; Sinclair *et al.*, 2007). *T. molitor* is one of resource insects. It is very nutritious, easy to culture, and has low dispersal ability and no hidden ecological danger. Nowadays it has been widely applied into agriculture (Huang *et al.*, 2005), animal husbandry, food, medical care (Zhang *et al.*, 2009), research, *etc.* At present the mealworm beetle is bred in many countries and new industries that raise and utilize *T. molitor* have emerged in some regions. The resistance of *T. molitor* to unfavorable environments is critical for the maintenance of a healthy and sustainable mealworm beetle industry. In order to study the mechanism of stress-resistance that could be applied towards the breeding of *T. molitor*, an *hsp*70 gene was cloned and analyzed from mealworm beetle larvae. The mRNA levels of the *hsp*70 gene of the beetle in different developmental stages and after heat shock were characterized, too.

2 MATERIALS AND METHODS

2.1 Insects

T. molitor larvae were from the Forest Protection Laboratory of Sichuan Agricultural University in China. They were fed on bran and supplement feed such as seasonal vegetables including carrot, lettuce leaves and Chinese cabbage, and small dried fish. All insects were cultured at 25℃, 60% RH and 12L∶12D photoperiod for approximately two years.

2.2 Genomic DNA isolation

Six 60-day old larvae of *T. molitor* that were starved for 3 days were stored in liquid nitrogen for later use. Genomic DNA was isolated from whole insect bodies using 2 × CTAB. DNA concentration and purity were determined by 0.8% agarose gel electrophoresis and UV-Vis spectrophotometer (DU® 800 Spectrophometer, Beckman Coulter) and the DNA sample was stored at −20℃ until further use.

2.3 Total RNA isolation

Six 60-day old larvae of *T. molitor* exposed to 42℃ for 1 h were stored in liquid nitrogen for use. Total RNA was extracted from whole insect bodies using RNAiso Plus Kit (TaKaRa). RNA concentration and purity were determined by 1.0% agarose gel electrophoresis and UV spectrophotometer and the sample was stored at −70℃ for future use.

2.4 Cloning of *hsp*70 core sequence from *T. molitor*

A pair of primers (P1/P2) (Table 1) for the amplification of *hsp*70 from *T. molitor* were designed based on the conserved regions in *Tribolium castaneum* HSP70 mRNA (GenBank accession: XM_968428) and *Anatolica polita borealis* HSP70

mRNA (GenBank accession: EF569673).

Several PCR reactions were performed using *T. molitor* genomic DNA as the template and various primer combinations (P1/P2). The reaction conditions included 94℃ for 4 min, 30 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 1 min, and a final extension at 72℃ for 10 min. Amplified products were detected on a 1.0% agarose gel and the target fragment was cloned into pMD20-T vector and transformed into *Escherichia coli* DH5α. Positive clones were sequenced by Shenzhen Huada Gene Research Institute and analyzed using BLAST program in the NCBI database.

Table 1　Primers used in this study

Primer name	Primer sequence (5′-3′)	Primer use
P1	GAGATCATCGCCAACGACCAAG	*hsp*70 core sequence PCR
P2	TGCGTTCGATGATCTTCGTCAT	
5′ RACE adaptor	AAGCAGTGGTATCAACGCAGAGTACGCGGG	Ligation of 5′ RACE
5′ RACE outer primer	CATGGCTACATGCTGACAGCCTA	Outer PCR of 5′ RACE
GSP1	GCCGTTGAAGTTGTTCTGGATGAGT	
5′ RACE inner primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	Inner PCR of 5′ RACE
GSP2	TAGCGTTGGTTCTCAAGTCCT	
3′ RACE outer primer	GAGATCATCGCCAACGACCAAG	Outer PCR of 3′ RACE
3′ RACE adaptor	GGAGATTAAGGGTAGGAGCTTTTTTTTTTTTTTTTT	Adaptor reaction of 3′ RACE
3′ RACE inner primer	TCCACTCGCATACCCAA	Inner PCR of 3′ RACE
3′ RACE downstream primer	GGAGATTAAGGGTAGGAGC	
Oligo d (T)	TTTTTTTTTTTTTTTTT	RT for SQ-PCR
P3	AAGGAAGCAAACTGTCGCCT	
P4	ATTCCCGCACTTTGTCCTCC	SQ-PCR
P5	AGCAAGAGAGGTATCCTCAC	
P6	ATCTCCTGCTCGAAGTCGAG	

2.5　5′RACE

For 5′RACE PCR, specific primers including GSP1 and GSP2 were designed from the beginning of the isolated *hsp*70 core sequence besides the 5′ RACE adaptor primer, outer and inner primers provided by the 5′-Full RACE Kit (TaKaRa) (Table 1). A series of reactions including dephosphorylation, decapping, ligation with 5′ RACE adaptor and reverse transcription were performed according to the instruction manual. After reverse transcription, nested PCR was carried out under two different PCR conditions. Cycling condition for outer PCR consisted of 94℃ for 3 min, 30 cycles of 94℃ for 30 s, 56℃ for 30 s and 72℃ for 3 min, and a final extension at 72℃ for 10 min. The inner PCR conditions were: 94℃ for 3 min, 32 cycles of 94℃ for 30 s, 55℃ for 35 s and 72℃ for 90 s, and a final extension at 72℃ for 10 min. Amplified DNA fragments were detected and fragments of the predicted size were subcloned into pMD20-T vector and transformed into *E. coli* DH5α. Positive clones were sequenced by Shenzhen Huada Gene Research Institute.

2.6　3′RACE

The isolated *hsp*70 core sequence was used to design a set of 3′ RACE primers including the adaptor primer, downstream primer, outer and inner primers (Table 1). First strand cDNA was synthesized using 1 μg total RNA as the template, and the outer PCR was carried out in a 15.0 μL reaction volume. Reaction parameters were as follows: 4 min at 94℃, 25 cycles of 30 s at 94℃, 30 s at 55℃ and 150 s at 72℃. The adaptor reaction conditions consisted of 94℃ for 4 min, 5 cycles of 94℃ for 30 s, 40℃ for 30 s and 72℃ for 90 s. The last or inner PCR conditions included 94℃ for 4 min, 38 cycles of 94℃ for 30 s, 52℃ for 30 s and 72℃ for 90 s, and a final extension of 72℃ for 10 min. Amplified products from the inner PCR were detected and the target fragments were cloned into pMD20-T vector and transformed into *E. coli* DH5α. Positive clones were sequenced by Shenzhen Huada Gene Research Institute.

2.7　Sequence analysis of *hsp*70 from *T. molitor*

The core sequence, 5′ terminal and 3′ terminal sequences of *hsp*70 from *T. molitor* were spliced together using EditSeq software of DNASTAR package. To verify whether the complete sequence of

*hsp*70 from *T. molitor* was obtained, specific PCR to amplify the full sequence was performed and the target products were sequenced. Nucleotide sequence homology was analyzed using BLASTN, and the ORF, coding region and deduced amino acid sequence were analyzed using the ORF Finder. The observed divergence homology tree based on the amino acid sequences of HSP70 from *T. molitor* and other insects was constructed by DNAMAN6.0.

2.8 Prediction of the physico-chemical property and structure of the deduced HSP70 from *T. molitor*

The amino acid composition, isoelectric point and molecular weight of the deduced HSP70 encoded by *hsp*70 from *T. molitor* were predicted using the expasy/protparam and expasy/compute pI/MW program on SwissProt. Signal peptide and transmembrane domains in the HSP70 were predicted using cbs/signalP, cbs/TMHMM-2.0 and ch.emb/TMpred respectively. The secondary structure, structural domain and tertiary structure were predicted using npsa/gor4, ebi/interproscan and expasy/swissmodel, respectively.

2.9 Semi-quantitative RT-PCR

Different day-old larvae, pupae and adults exposed to 42℃ for 1 h and corresponding controls at 25℃ were comparatively analyzed by semi-quantitative RT-PCR (SQ-PCR). The larvae tested were 30, 60 and 90 day-old from egg hatch. The pupae used in the test were 1 and 5 day-old after pupation from larvae. The adults tested were 1, 15 and 30 day-old after emergence from pupae. Insects at different developmental stages were immediately frozen in liquid nitrogen after exposure to 42℃ and 60% RH for 1 h in environmental growth chambers. A control was prepared and handled similarly, using insects of different developmental stages exposed to 25℃ and 60% RH for 1 h. Total RNA was extracted from whole insect bodies using RNAiso Plus Kit and was further cleaned using an RNeasy Minielute Cleanup kit (Qiagen). The quality and concentration were determined by an UV/visible spectrophotometer. The integrity of the RNA was confirmed by formaldehyde agarose gel electrophoresis. Total RNA from each stage was checked for genomic DNA contamination by PCR amplification of 1 μL RNA sample by using gene-specific primers for *hsp*70. The amplified products were analyzed on 2% agarose gel containing ethidium bromide. SQ-PCR was performed to further compare mRNA expression levels of *hsp*70 gene in *T. molitor* at different developmental stages.

First-strand cDNAs were synthesized for SQ-PCR by using total RNA. All RNA samples from different developmental stages were subjected to reverse transcription simultaneously. The first-strand cDNA reaction was aliquoted and stored at −20℃ for later use.

SQ-PCR was carried out in a final volume of 20 μL reaction mixtures by using *hsp*70 gene-specific primers P3 and P4 and house-keeping gene *β-actin* primers P5 and P6 (Table 1). The PCR conditions for *β-actin* and for *hsp*70 were identical as follows: 94℃ for 3 min, 25 cycles at 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, and a final extension at 72℃ for 10 min. The SQ-PCR was repeated three times with total RNA extracted from three groups of different day-old larvae, pupae and adults of *T. molitor*. The amplified products were detected on a 2% agarose gel containing ethidium bromide and the images were collected by using Gel Doc XR + (Bio-Rad, USA). The intensities of gel electrophoresis images collected were determined by using Quantity One® (version 4.6.2, Bio-Rad, USA). Band intensity of *β-actin* mRNA from each group was given a value of 100%. The relative abundance of *hsp*70 mRNA from each group was expressed as a percentage of band intensity of *hsp*70 to *β-actin*.

2.10 Data statistics and analysis

Quantified intensities of SQ-PCR were subjected to two-way analysis of variance (ANOVA) of SPSS17.0 to determine significant differences in *hsp*70 mRNA expression levels among different developmental stages and two temperatures. Significant differences among developmental stages within a temperature treatment recognized by the two-way ANOVA were determined by Tukey's multiple range test ($P<0.05$). The diagram was drawn with Microsoft Excel software.

3 RESULTS

3.1 Cloning and sequencing of *hsp*70 from *T. molitor*

A PCR fragment 1 097 bp in length was obtained from the genomic DNA of *T. molitor*. Sequence alignments using BLAST showed that the isolated sequence shares 83% nucleotide sequence identity with *Microdera dzhungarica punctipennis hsp*70 (GenBank accession: JF421286) and 82% with *T. castaneum hsp*70 (GenBank accession: XM_969349), suggesting that the obtained sequence is the core sequence of *hsp*70 gene from *T. molitor*. The amplified sequence was therefore named *Tmhsp*70. The 5′ and 3′ ends of *Tmhsp*70 core sequence were then used to design specific primers

for 5′ and 3′ RACE, respectively. A 5′ sequence of 803 bp and a 3′ sequence of 1 093 bp were acquired by 5′ and 3′ RACE respectively, and a 2 282 bp full length sequence was assembled by sequence splicing. The complete sequence obtained was verified by specific PCR and sequencing. The full length sequence of *Tmhsp*70 shares 81% nucleotide sequence identity with *M. dzhungarica punctipennis hsp*70 and 82% with *T. castaneum hsp*70, respectively. *Tmhsp*70 sequence was submitted to GenBank and assigned the accession number JQ219848.

The sequence of *Tmhsp*70 contains a 115 bp 5′ untranslated region (5′ UTR) rich in adenine residues, a 232 bp 3′ untranslated region (3′ UTR) rich in adenine and thymine residues and a 1 935 bp open reading frame (116 – 2 050 bp) which encodes 644 aa (Fig. 1). As shown in Fig. 1, the deduced HSP70 encoded by *Tmhsp*70 contains three signature motifs of HSP70 family, *i. e.*, IDLGTTYS (9 – 16 residues), IFDLGGGTFDVSIL (197 – 210 residues) and IVLVGGSTRIPKIQQ (335 – 349 residues), as well as a EEVD (641 – 644 residues) motif characteristic of cytoplasmic HSP70s. In addition, *Tmhsp*70 has seven repeats of the heat shock element (HSE) motif nGAAn in its 5′ UTR, a 22 bp Poly (A) tail at the 3′ UTR and a polyadenylation signal (PAS) at 2 242 – 2 247 bp.

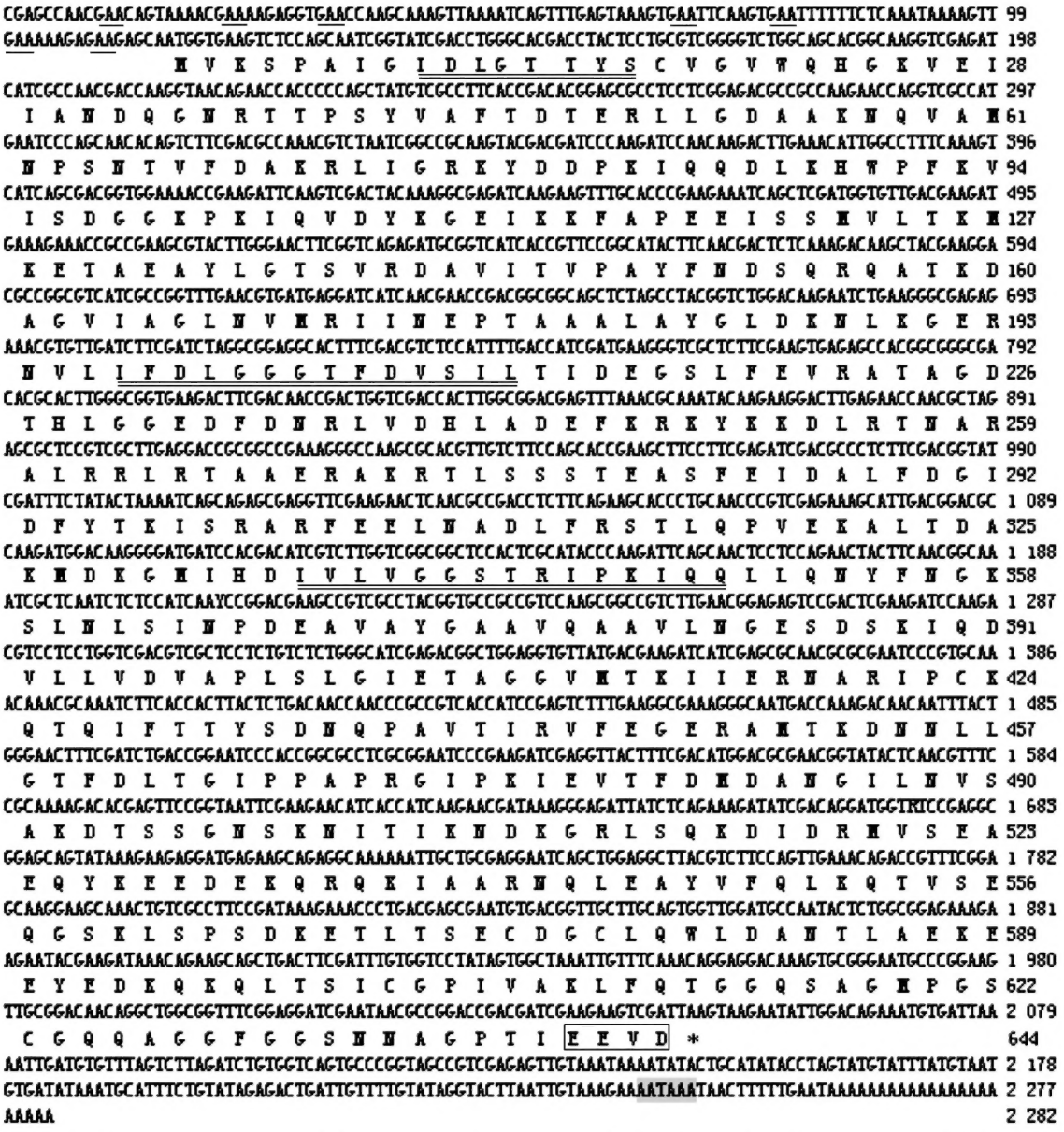

Fig. 1　The nucleotide and the deduced amino acid sequences of a *hsp*70 gene from *Tenebrio molitor* (*Tmhsp*70)

Numbers on the right represent numbers of bases or amino acid residues, heat shock elements are underlined, signature motifs of HSP70 family are indicated by double underline, cytoplasmic HSP70 signature motif is boxed, termination codon is indicated by asterisk, and polyadenylation signal is shadowed.

Amino acid sequence homology between HSP70s from *T. molitor* and other insects is very high as shown in Fig. 2. The deduced sequence of HSP70 (named as TmHSP70) encoded by *Tmhsp*70 shares the highest homology with HSP70s from three other beetles including *T. castaneum*, *M. dzhungarica punctipennis* and *A. polita borealis*.

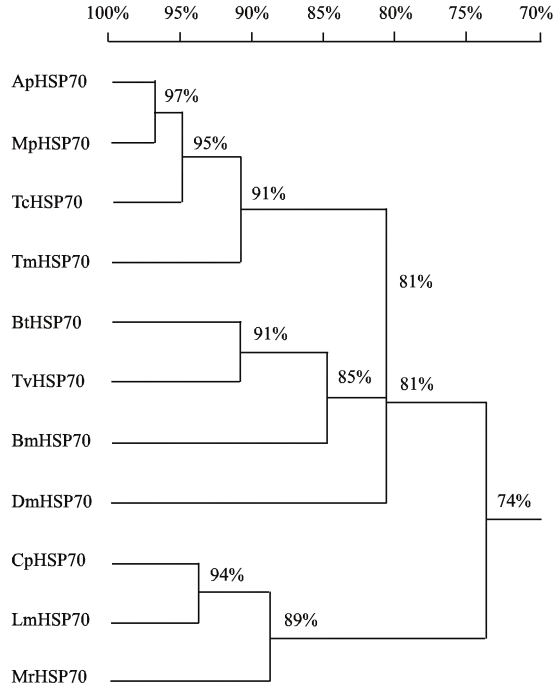

Fig. 2 Observed divergence homology tree based on the amino acid sequences of HSP70 from *Tenebrio molitor* and other insects

ApHSP70: *Anatolica polita borealis* HSP70 (ABQ39970); MpHSP70: *Microdera dzhungarica punctipennis* HSP70 (AEB52075); TcHSP70: *Tribolium castaneum* HSP70 (XP_974442); TmHSP70: *Tenebrio molitor* HSP70 (AFE88579); BtHSP70: *Bemisia tabaci* HSP70 (ACZ52196); TvHSP70: *Trialeurodes vaporariorum* HSP70 (ACH85201); BmHSP70: *Bombyx mori* HSP70 (BAF69068); DmHSP70: *Drosophila melanogaster* HSP70 (NP_731716); CpHSP70: *Cryptocercus punctulatus* HSP70 (AFK49798); LmHSP70: *Locusta migratoria* HSP70 (AAO21473); MrHSP70: *Megachile rotundata* HSP70 (AAS57864).

3.2 Physico-chemical properties of the deduced HSP70 from *T. molitor*

The main physico-chemical properties of the deduced TmHSP70 from *T. molitor* were analyzed using expasy/protparam and expasy/compute pI/MW programs at SwissProt and the results are shown in Table 2. The relative molecular mass of TmHSP70 is 70.5 kDa with 644 total amino acids among which ninety are negatively charged and eighty are positively charged. Among the total amino acids, the contents of Ala, Gly and Leu are relatively higher and account for 8.9%, 8.1% and 7.9% of the protein, respectively; meanwhile, the contents of Cys, His and Trp are relatively lower and only account for 0.9%, 0.8% and 0.5%, respectively. The isoelectric point of TmHSP70 is about 5.4 and grand average of hydropathicity is −0.423, indicating the hydrophilic and weak acidic nature of the protein. In addition, the predicted TmHSP70 has no signal peptide or transmembrane domains.

3.3 Secondary structure and structural domain of deduced HSP70 from *T. molitor*

The secondary structure of the deduced TmHSP70 consists of multiple alpha helices (39.91%), beta sheets (16.93%) and random coils (43.17%) (Fig. 3).

The deduced TmHSP70 has a highly conserved N-terminal ATPase domain of ca. 42 kDa (3 – 383 residues) and a conserved C-terminal domain of ca. 26 kDa (389 – 619 residues) which is further subdivided into a conserved peptide-binding subdomain of ca. 18 kDa (389 – 544 residues) and a non-conserved indeterminate C-terminal subdomain of ca. 9 kDa (538 – 619 residues) (Fig. 4).

3.4 Tertiary structure of the deduced HSP70 from *T. molitor*

The tertiary structure prediction using expasy/swissmodel in SwissProt indicated that the ATPase domain (3 – 383 residues) of TmHSP70 consists of two large globular subdomains (Ⅰ and Ⅱ) (Fig. 5: A) which are separated by a deep cleft in the middle and connected by two crossed alpha helices, Each large globular subdomain is further divided into two small subdomains. The four subdomains (Ia, Ib, IIa and IIb) (Fig. 5: B) and the two crossed alpha helices form the nucleotide-binding core at the bottom of the middle cleft. Nucleotide binds in complex with one kalium ion contacting all four subdomains.

Table 2 Physico-chemical properties of the deduced HSP70 from *Tenebrio molitor*

Protein	Relative molecular weight (kDa)	Molecular formula	Number of negatively charged amino acids	Number of positively charged amino acids	Total number of amino acids	Content of representative amino acids (%)						pI	Grand average of hydropathicity
						Amino acids of higher content			Amino acids of lower content				
						Ala	Gly	Leu	Cys	His	Trp		
TmHSP70	70.5	$C_{3085}H_{4962}N_{862}O_{986}S_{18}$	80	90	644	8.9	8.1	7.9	0.9	0.8	0.5	5.4	−0.423

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AA        MVKSPAIGIDLGTTYSCVGVWQHGKVEIIANDQGNRTTPSYVAFTDTERLLGDAAKNQVAMNPSNTVFDA
Pre-sec   ccccceeeeeceeeeeeeecccccceeeeeccccccccceeeeccchhhhhhhhhhhhhcccccchhhh
AA        KRLIGRKYDDPKIQQDLKHWPFKVISDGGKPKIQVDYKGEIKKFAPEEISSMVLTKMKETAEAYLGTSVR
Pre-sec   hhhhccccccchhhhhccccceeeeccccceeeeccccccccchhhhhhhhhhhhhhhhhhhhcccccc
AA        DAVITVPAYFNDSQRQATKDAGVIAGLNVMRIINEPTAAALAYGLDKNLKGERNVLIFDLGGGTFDVSIL
Pre-sec   ceeeeecccccchhhhhhhhhccccchhhhhhhcchhhhhhhhhhhhcccccceeeeeeccccccceeeee
AA        TIDEGSLFEVRATAGDTHLGGEDFDNRLVDHLADEFKRKYKKDLRTNARALRRLRTAAERAKRTLSSSTE
Pre-sec   ecccccchhhhhhccccccccccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccccch
AA        ASFEIDALFDGIDFYTKISRARFEELNADLFRSTLQPVEKALTDAKMDKGMIHDIVLVGGSTRIPKIQQL
Pre-sec   hhhhhhhcccccchhhhhhhhhhhhhhhhhhhhhcccchhhhhhhhhhhhcccccceeeeeccccccchhhhhh
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Pre-sec   hhhcccccceeccccccchhhhhhhhhhhhhhccccccceeeeeeeeccccccccceeccccchhhhhhhccc
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Pre-sec   ccccccccccceeeeeccccccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccc
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Pre-sec   ccccccceeccccccchhhhhhcchhhhhhhhhhhhceeeccccceeeeeccccccccccccccccccccc
AA        GGSNNAGPTIEEVD
Pre-sec   ccccccccceeeeec
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Fig. 3　Secondary structure prediction of the deduced HSP70 from *Tenebrio molitor*

AA: Amino acid sequence; Pre-sec: Predicted secondary structure; h: Alpha helix; e: Beta sheet; c: Random coil.

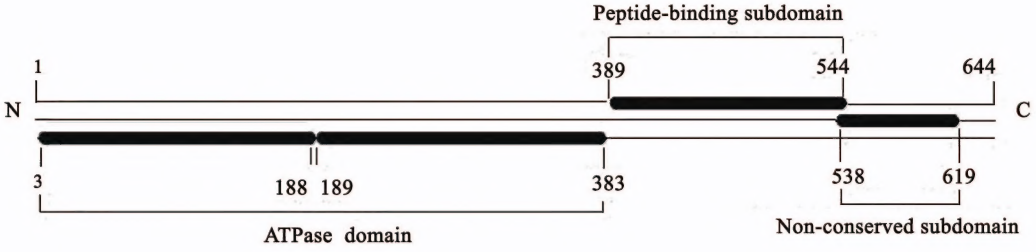

Fig. 4　Domains of deduced HSP70 from *Tenebrio molitor*

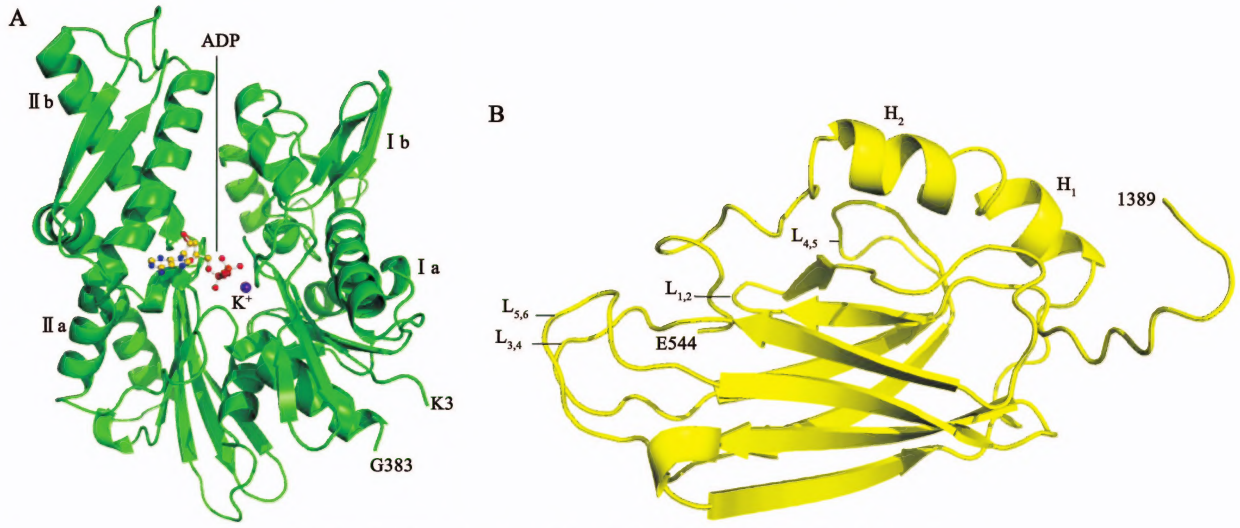

Fig. 5　Tertiary structure of the deduced HSP70 from *Tenebrio molitor*

A: ATPase domain; B: Peptide-binding domain, $L_{1,2}$, $L_{3,4}$, $L_{4,5}$ and $L_{5,6}$ are four loops, and H_1 and H_2 are two α-helices.

The peptide binding moiety (residues 389 – 544) forms a sandwich of 2 four-stranded β-sheets with four loops protruding upwards (two inner and two outer loops) and two α-helices (H_1 and H_2) (Fig. 5: A) which are packed against the inner loops $L_{1,2}$ and $L_{4,5}$ (Fig. 5: B). The substrate binding cavity is formed by the β-sheets 1 and 2 and the loops $L_{1,2}$ and $L_{3,4}$. The helix (H_1) constitutes a lid which covers the

cavity to prevent the escape of peptide substrates that bind.

3.5 Expression levels of *Tmhsp*70 determined using semi-quantitative PCR

A low level of *Tmhsp*70 mRNA expression was detected in all life stages at 25℃ while a dramatic increase in expression of *Tmhsp*70 was detected in all developmental stages of heat-shocked insects (42℃) (Fig. 6). The densitometric analysis of the gel images for SQ-PCR revealed that the *Tmhsp*70 expression was significantly different between the two temperatures ($F = 15,068.91$; $df = 1, 31$; $P = 0.000$; $n = 3$) and among different developmental stages ($F = 715.72$; $df = 7, 31$; $P = 0.000$). The interaction between temperature and stage was significant, too ($F = 356.59$; $df = 7, 31$; $P = 0.000$). The expression of *Tmhsp*70 increased by 1.4 – 26.9-fold in the heat-shocked developmental stages of *T. molitor* (42℃), compared with that of the control (25℃) (Fig. 6: B). It seems clear that *Tmhsp*70 is induced by heat shock.

In addition, the expression of *Tmhsp*70 mRNA in *T. molitor* is dependent upon development. The relative abundance of *Tmhsp*70 mRNA in 1-day old pupae was the highest among the control groups (25℃), and within the heat-shocked groups (42℃), *Tmhsp*70 mRNA in the 90-day old larvae became the most abundant (Fig. 6: B). *Tmhsp*70 mRNA expressed constitutively in 1-day old pupae was higher than that accumulated in other developmental stages at 25℃; meanwhile *Tmhsp*70 mRNA inducibly expressed in 90-day old larvae was higher than that accumulated in 30- and 60-day old larvae or accumulated in 15- and 30-day old adults after exposure to 42℃ for 1 h.

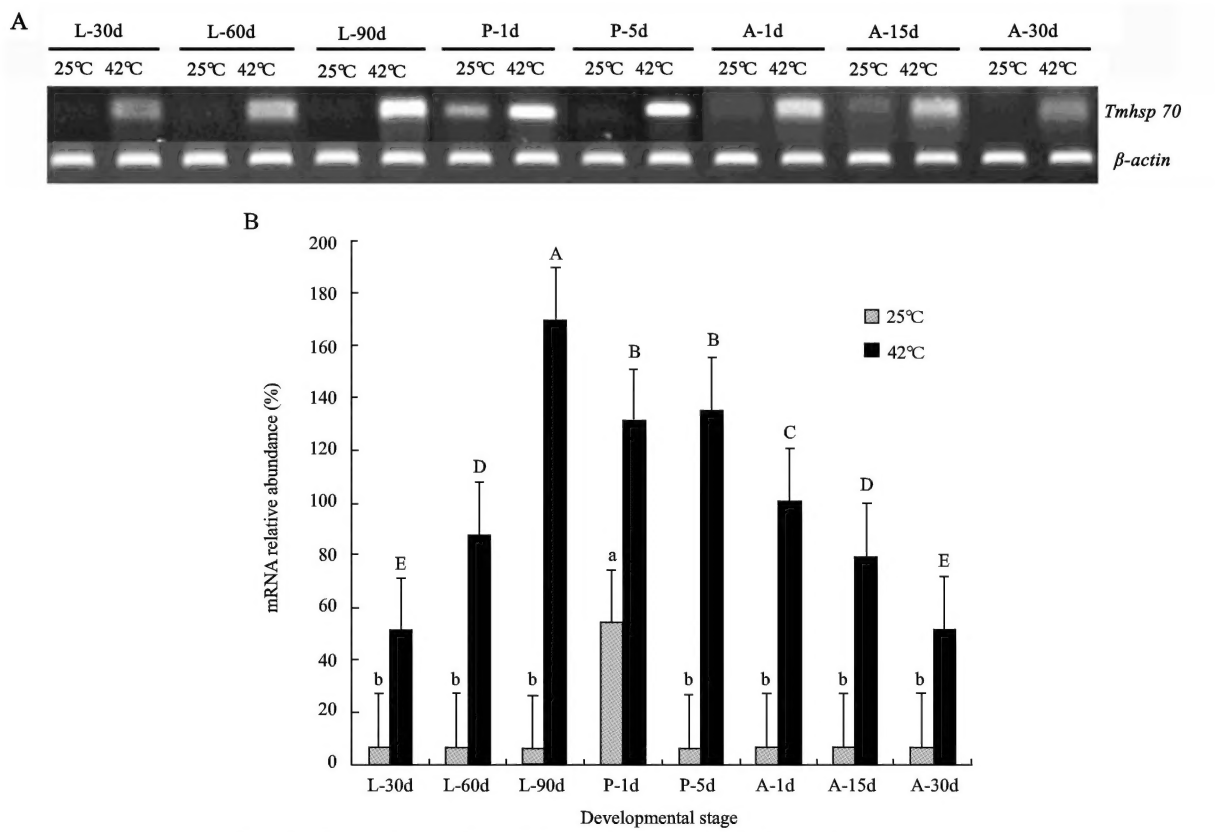

Fig. 6 Expression profiles of *Tmhsp*70 in *Tenerio molitor* during development

A: Semi-quantitative RT-PCR electropherogram; B: Relative abundance of *Tmhsp*70 mRNA in the control (25℃) and heat-shocked (42℃) insects at different developmental stages. L-30d, L-60d and L-90d are 30-, 60- and 90-day old larvae from egg hatch, respectively; P-1d and P-5d are 1- and 5-day old pupae after pupation, respectively; A-1d, A-15d and A-30d are 1-, 15- and 30-day old adults after emergence, respectively. Different letters above bars within a temperature indicate significant difference ($P < 0.05$, Tukey's test).

4 DISCUSSION

4.1 Sequence characteristics and function of *Tmhsp*70

A 2 282 bp full sequence of *hsp*70 from *T. molitor* (*Tmhsp*70) was cloned by PCR and rapid amplification of 5′ and 3′ cDNA ends. Nucleotide sequence analysis indicated that it contains a 115 bp 5′ untranslated region (5′ UTR) rich in adenine, a

1 935 bp open reading frame, a 232 bp 3′ untranslated region (3′ UTR) rich in adenine and thymine. It has seven repeats of the heat shock element nGAAn in its 5′ UTR. The heat shock elements (HSE) are responsible for regulating heat shock gene expression in eukaryotes (Schlesinger, 1990). For maximum heat shock induction, a functional HSE should contain a minimum of three contiguous nGAAn units and two HSEs positioned close to the transcriptional start site (Papadimitriou *et al.*, 1998). The unique 5′ and 3′ untranslated regions have critical effect on the regulation of stability and translation of *hsp* mRNAs (McGarry and Lindquist, 1985; Lee, 1998).

Amino acid sequence analysis indicated that the TmHSP70 encoded by *Tmhsp*70 has neither a signal peptide nor a transmembrane domain and it shares some common structural features with other HSP70s studied so far. Its secondary structure includes two main functional domains: a 42 kDa highly conserved N-terminal ATPase domain and an 18 kDa conserved C-terminal peptide-binding domain. The tertiary structure of ATPase domain is composed of two large globular subdomains and contains a nucleotide-binding core. Tertiary structure of the peptide-binding domain forms a sandwich of 2 four-stranded β-sheets and two α-helices, and includes a peptide-binding cavity. Together the two domains form the molecular foundation for the TmHSP70 chaperone activity. As molecular chaperones, HSP70s not only participate in the folding and transportation of newly translated peptides but also can repair misfolded proteins or accelerate degradation of some proteins that are unable to restore their native conformation. This function protects the normal physiological processes of cells (Feder and Hofmann, 1999).

4.2 Expression profiles of *Tmhsp*70

The mRNA expression of *Tmhsp*70 from different developmental stages of *T. molitor* was determined by semi-quantitative RT-PCR. The results revealed that the *Tmhsp*70 mRNA was expressed constitutively at a very low level in *T. molitor* (25℃) and its expression was sharply and dramatically up-regulated when insects were exposed to an elevated temperature (42℃). The overall increase in the levels of *Tmhsp*70 mRNA in different life stages ranged from 1.4- to 26.9-fold on the basis of semi-quantitative RT-PCR analysis, and *Tmhsp*70 mRNA inducibly expressed in 90-day old larvae became the most abundant and was higher than that accumulated in 30- and 60-day old larvae or accumulated in 15- and 30-day old adults. The up-regulated *Tmhsp*70 mRNA may be contributing to the increased thermotolerance of the elder larvae.

Furthermore, expression of *Tmhsp*70 mRNA has the characteristics of developmental regulation. *Tmhsp*70 mRNA expressed constitutively in the 1-day old pupae (P-1d) was more abundant than accumulated in other tested developmental stages including larvae (L-30d, L-60d and L-90d), old pupae (P-5d) and adults (A-1d, A-15d and A-30d). The *Tmhsp*70 mRNA expressed abundantly in the 1-day old pupae may be directly connected with the physiological characteristics of the 1-day old pupae. For the 1-day old pupae, lots of physiological and biochemical processes, such as cuticle sclerotization, larval tissues dissociation and imaginal tissues construction remain to be completed, and there are a large number of proteins to be synthesized, degraded and transported. The relative rich mRNA of *Tmhsp*70 expressed may lead TmHSP70 to synthesize in abundance. As molecular chaperones, the richer TmHSP70s synthesized in the 1-day old pupae may be involved in the folding and transporting of nascent peptides and assembly, aggregation and degradation of certain proteins to regulate the biological functions of some related proteins and vital processes.

Previous researches showed that HSP70s were not only expressed in cells exposed to heat stress but also constitutively expressed in all living cells including nuclei, cytoplasts, endoplasmic reticula, mitochondria, chloroplasts, *etc.* (Wu *et al.*, 2004). The regulation of HSP gene expression occurred primarily at the transcriptional level (Lindquist, 1986) and the mRNA coding for the protein can be induced about 1- to 1 000-fold (Lindquist, 1986). The expression profiles of *Tmhsp*70 in different cells and organelles of *T. molitor* are unknown, and the regulation of the gene expression is unclear, too.

4.3 Further research on HSP genes from *T. molitor*

In this study we cloned and analyzed a complete sequence of *hsp*70 from *T. molitor* larvae for the first time, and determined the mRNA relative abundance in the beetle life stages exposed to two different temperatures. Our results have laid a foundation for further studying structure, function and expression regulation of HSPs from *T. molitor* as well as to determine the relationship between HSPs and stress-resistance in the beetle. But more expression profiles of *Tmhsp*70 are still to be further determined, such as the specific expression in different tissues exposure to heat and other stresses including cold, insecticides, pathogenic microorganisms, parasites, *etc.* Furthermore, previous reports suggest a

correlation between different body-colors observed on *T. molitor* individuals and stress-resistance (Huang *et al.*, 2011, 2012). Therefore, to clarify the mechanism of stress-resistance and improve variety breeding, further research on cloning, expression, structure and function of various HSP genes from different color varieties of *T. molitor* is necessary.

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黄粉虫热休克蛋白 70 基因的克隆、序列分析与表达

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摘要：为给黄粉虫 *Tenebrio molitor* 抗逆机理研究提供理论依据，本研究采用 PCR 和 RACE 法从黄粉虫幼虫中克隆出一个热休克蛋白 70 基因 *Tmhsp*70，并运用半定量 RT-PCR 法检测其在黄粉虫不同发育阶段的 mRNA 表达水平。结果表明：克隆出的 *Tmhsp*70 序列全长 2 282 bp，具有一个富含 A 的 115 bp 5′-非翻译区和一个 1 935 bp 的开放阅读框及一个富含 A、T 的 232 bp 3′-非翻译区。5′-非翻译区含有 7 个热休克元件 nGAAn，3′-非翻译区末端有长 22 bp 的 Poly(A)尾。*Tmhsp*70 编码的黄粉虫热休克蛋白(TmHSP70)具有 3 个典型的 HSP70 特征基序(IDLGTTYS，IFDLGGGTFDVSIL 和 IVLVGGSTRIPKIQQ)和 1 个胞质 HSP70 末端特征基序(EEVD)，无信号肽和跨膜区域，包含 2 个主要的结构域，即：N-端 42 kDa 的高度保守 ATPase 功能域和 C-端 18 kDa 的保守多肽结合功能域。ATPase 功能域的三级结构由 2 个大球形亚功能域组成，具有 1 个核苷酸结合中心；多肽结合功能域形成 1 个双层 4 股 β-折叠片样的三明治结构和 2 个 α-螺旋，内含 1 个多肽结合通道。此外，黄粉虫 *Tmhsp*70 mRNA 的表达具有热激诱导和发育调控的特征。半定量 RT-PCR 分析表明，42℃热激 1 h 的黄粉虫各发育阶段 *Tmhsp*70 mRNA 的表达量上升了 1.4～26.9 倍。25℃下 1 日龄黄粉虫蛹中的 *Tmhsp*70 mRNA 表达量要高于其余各发育阶段的累积表达量；42℃热激 1 h 后 90 日龄幼虫中的 *Tmhsp*70 mRNA 表达量最丰富，既高于 30 日龄和 60 日龄幼虫中的累积表达量，也高于 15 日龄和 30 日龄成虫中的累积表达量。这些结果为进一步研究黄粉虫热休克蛋白的结构、功能和表达调控及其与抗逆性的关系奠定了基础。

关键词：黄粉虫；热休克蛋白；基因克隆；生物信息学；mRNA 表达；热激

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